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Cloning and analysis of the PTS-1 receptor in *Trypanosoma brucei*☆,☆☆

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Abstract

Kinetoplastid organisms, such as the protozoan parasite *Trypanosoma brucei*, compartmentalise several important metabolic pathways in organelles called glycosomes. Glycosomes are related to peroxisomes of yeast and mammalian cells. A subset of glycosomal matrix proteins is routed to the organelles via the peroxisome-targeting signal type 1 (PTS-1). The *PEX5* gene homologue has been cloned from *T. brucei* coding for a protein of the translocation machinery, the PTS-1 receptor. The gene codes for a polypeptide of 654 amino acids with a calculated molecular mass of 70 kDa. Like its homologue in other organisms *T. brucei* PTS-1 receptor protein (TbPEX5) is a member of the tetratricopeptide repeat (TPR) protein family and contains several copies of the pentapeptide W-X-X-X-F/Y. Northern and Western blot analysis showed that the protein is expressed at different stages of the life cycle of the parasite. The protein has been overproduced in *Escherichia coli* and purified using immobilized metal affinity chromatography. The purified protein specifically interacts *in vitro* with glycosomal phosphoglycerate kinase-C (PGK-C) of *T. brucei*, a PTS-1 containing protein. The equilibrium dissociation constant (K_d) of PGK-C for purified TbPEX5 is 40 nM. Using biochemical and cytochemical techniques a predominantly cytosolic localization was found for TbPEX5. This is consistent with the idea of receptor cycling between the glycosomes and the cytosol.

Abbreviations: HK, hexokinase; ORF, open reading frame; PCR, polymerase chain reaction; PEX5, peroxin 5; PGK-C, glycosomal phosphoglycerate kinase; PTS-1, peroxisome-targeting signal type-1; TbPEX5, *T. brucei* PTS-1 receptor protein; TPR, tetratricopeptide repeat.

☆ *Note*: Nucleotide sequence data reported in this paper are available in the GenBank, EMBL and DDBJ databases under the accession number AF142475.

☆☆ Throughout this paper, the genetic nomenclature as proposed for trypanosomatids has been applied [55], not only for *T. brucei*, but, in the interest of clarity, also for human and yeasts, although the convention for yeasts is somewhat different (protein: Pex5p instead of PEX5) [56].

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1. Introduction

Trypanosoma brucei, the causative agent of African sleeping sickness in man and the related disease Nagana in various domestic animals, compartmentalises the major part of the glycolytic pathway in an organelle, hence called glycosome. Glycosomes were also found in all other members of the protozoan Kinetoplastida order examined. The glycosomes of *T. brucei* may contain in addition to the enzymes involved in glycolysis also some enzymes of glycerol metabolism, fatty-acid β-oxidation, ether-lipid biosynthesis and pyrimidine synthesis [1,2].

The enzymatic contents of the glycosomes varies during the life cycle of the trypanosomes. In *T. brucei* bloodstream form, the role of the organelle is mainly in glycolysis: the nine enzymes involved in the conversion of glucose into 3-phospho-glycerate and, under anaerobiosis, also glycerol constitute more than 90% of the organelle's protein content. In cultured trypanosomes, representative of the procyclic trypomastigote living in the midgut of the tsetse fly, the level of the majority of the glycosomal enzymes involved in glycolysis is decreased [3]. These variations in the composition of the glycosome tally with the change in the overall metabolic activity of the parasite. Bloodstream-form trypanosomes are for the synthesis of ATP entirely dependent on the conversion of glucose into pyruvate. They lack a functional Krebs' cycle and the mitochondrial system for oxidative phosphorylation. The procyclic forms rely more on mitochondrial activity. Substrates other than glucose, such as amino acids and fatty acids, are then also used [4].

Glycosomes are organelles closely related to peroxisomes of other eukaryotes. They have a similar morphology and equilibrate at a comparable density during isopycnic centrifugation. Even if their enzymatic content is very different, several enzymes such as those involved in fatty-acid β-oxidation, ether-lipid biosynthesis or pyrimidine biosynthesis are observed in both glycosomes and peroxisomes. Catalase, the hallmark enzyme of peroxisomes, is never found in the glycosomes of *T. brucei*, but it is present in the organelles of the related kinetoplastid organisms *Crithidia* and *Phytomonas* [5–7]. The biogenesis of both kinds of organelles is also similar. Glycosomes, like peroxisomes, do not contain any DNA. All their matrix proteins are encoded on nuclear chromosomes, synthesized on free ribosomes in the cytosol, and imported post-translationally into the organelles [8,9].

The relationship between glycosomes and peroxisomes is further supported by the finding that they use similar sequences for the routing of their polypeptides. Two types of peroxisome-targeting signals (PTS) have been recognized [10]. PTS-1 is a C-terminal tripeptide: -SKL or a variant thereof. This signal has been demonstrated to be also functional in *T. brucei* [11,12]. The majority of the glycosomal enzymes sequenced so far has a PTS-1 type motif. PTS-2 is an N-terminal nonapeptide usually starting no more than ten residues from the initiator methionine, with a loosely conserved sequence motif. So far, this signal has only been detected in aldolase and hexokinase (HK) of *T. brucei* [13,14].

It has been shown in yeast and human cells that newly synthesized proteins with a PTS-1 are first recognized in the cytosol by a soluble receptor called peroxin 5 or PEX5. The receptor targets them, via interaction with the membrane peroxins PEX13 and PEX14, to the import machinery at the surface of the peroxisome [15]. The PTS-1 containing polypeptide is then translocated to the peroxisomal matrix.

In this paper, the cloning of the gene encoding the PEX5 homologue of *T. brucei* is described and the results of a preliminary analysis of its role in glycosome biogenesis are reported.

2. Materials and methods

2.1. Materials

Restriction endonucleases and modifying enzymes were purchased from Boehringer Mannheim (Germany) or New England Biolabs (USA). The TALON resin for the purification of $(His)_6$-tagged protein was from Clontech (USA). Oligonucleotide primers were obtained from Eurogentec (Belgium).

2.2. Cloning of the PEX5 gene

Polymerase chain reaction (PCR) amplification was performed on *T. brucei* genomic DNA using two degenerate oligonucleotides: 5′-TCCGAGGCYGSYCTSGCYTTYGARGCNGC-3′, sense primer and 5′-TTGGCGAGRGHRGCRCCSAGNCKRTTCCA-3′, antisense primer. These degenerate oligonucleotides are based on two conserved motifs as observed in an amino-acid sequence alignment of PEX5 from *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris* and *Yarrowia lipolytica* [16–19]. The first conserved motif SEA(A/G)LAFEAA, corresponding to residues 367–375 in the alignment in Fig. 1, was used to design the sense primer. The second conserved motif WNRLGA(S/A/T)LAN, corresponding to residues 537–546, was used to design the antisense primer. PCR was performed using Goldstar Red DNA polymerase according to the instructions of the supplier (Eurogentec). A major amplified product of 550 bp was purified, ligated into pZErO-2 (Invitrogen, USA) and cloned for sequencing.

A genomic library of *T. brucei* in *Escherichia coli* MB406 with the vector GEM11 (Promega, USA) was screened with the cloned PCR fragment, using stringent conditions [20]. Positive plaques were purified and rescreened. High-titre phage lysates were prepared and DNA was purified from the phages as described [21].

2.3. Sequence determination and Southern blotting

A 3.615 kb *Nsi*I fragment from one of the hybridizing phages was subcloned into pZErO-2, and several shorter fragments of it were subsequently ligated into pBluescript SK or KS (Stratagene, USA). Sequencing was carried out using the Thermo Sequenase kit (Amersham, UK) and fluorescently labelled forward and reverse sequencing primers. Reactions were run on a LI-COR automated sequencer. All regions of the 3.615 kb fragment were sequenced at least once in both directions. The genomic organization of the *T. brucei* PEX5 gene and its copy number were studied by Southern blot analysis.

2.4. Northern blot analysis

Total RNA was isolated from *T. brucei* bloodstream and culture form as described [22]. A *Nde*I–*Sal*I fragment from plasmid pTbPEX5 containing the 5′ half of the coding region was radiolabeled with [α-^{32}P]dCTP by nick translation and used as a probe.

2.5. Expression and purification of recombinant PEX5 in E. coli

A polypeptide containing the C-terminal 505 amino acids of *T. brucei* PEX5 was overproduced in *E. coli* using a bacteriophage T7 RNA polymerase system [23]. The gene was ligated in the expression vector pET15b (Novagen, USA) after amplification, for which two oligonucleotides were synthesized. The resulting plasmid pTbPEX5 directs the production of a fusion protein with a $(His)_6$ tag at the N-terminus under the control of the T7 promoter. The *PEX5* gene was amplified using two oligonucleotides: (1) 5′-ATGAAC**ATGCAT**ATGGCGCCAGTAGCAACA-3′, a sense primer containing the sequence corresponding to the amino acids MAPVAT of the *PEX5* gene and, at the position of the start codon, an *Nde*I site (indicated in bold); (2) 5′-TTT**GGATCC**TCACGTAACATAGACTG-3′, an antisense primer complementary to the end of the gene and containing a *Bam*HI site (in bold). The *PEX5* gene was amplified using Vent DNA polymerase according to the instructions of the supplier (New England Biolabs, USA). The amplified fragment was purified, digested with *Nde*I and *Bam*HI, and ligated in pET15b to form the plasmid pTbPEX5.

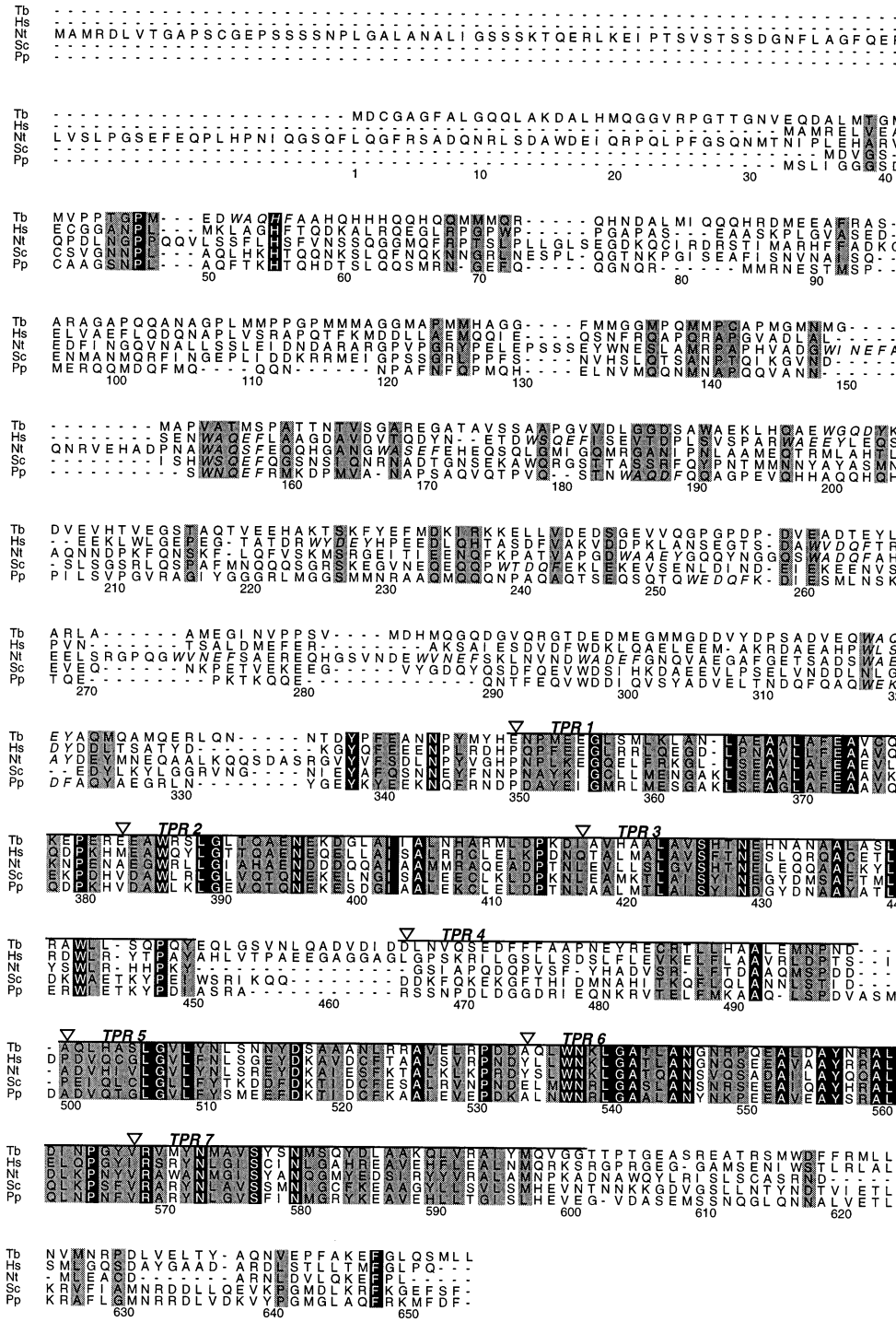

Fig. 1.

E. coli strain BL21(DE3) transformed with the plasmid pTbPEX5 was grown at 37°C in LB medium, supplemented with 100 μg ml^{-1} ampicillin. Expression was induced at an OD_{600nm} of 0.5–0.6 by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and growth was continued for another 2 h. Cells were collected by centrifugation (12 000 × *g*, 10 min, 4°C). The cell pellet was resuspended in 40 ml of cell lysis buffer containing 100 mM triethanolamine/HCl (pH 8.0), 250 mM NaCl, 10% glycerol and a protease inhibitor mixture (1 μM phenylmethylsulfonyl fluoride, and 1 mM each E-64, leupeptin and pepstatin). Cells were lysed by two passages through a SLM-Aminco French pressure cell (SLM Instruments, USA) at 13 000 psi. The lysate was diluted 2.5x with lysis buffer and centrifuged (12 000 × *g*, 15 min, 4°C). The soluble cell fraction was used to further purify $(His)_6$-PEX5. First, nucleic acids were degraded by incubation in the presence of 500 units Benzonase (Merck, Germany) for 30 min at 20°C. Then $(His)_6$-PEX5 was precipitated from the supernatant by addition of 40% ammonium sulfate and stirring on ice for 20 min. The precipitate was collected by centrifugation (12 000 × *g*, 20 min, 4°C) and resuspended in lysis buffer. The protein was further purified using immobilized metal affinity chromatography (TALON resin, Clontech, USA) exploiting the $(His)_6$ tag at the N-terminus of the protein. The purity of the protein was assessed by SDS-PAGE followed by Coomassie brilliant blue staining.

2.6. Antibodies

Purified $(His)_6$-PEX5 was used to raise a polyclonal antiserum in rabbits. The antiserum was affinity purified by incubation with $(His)_6$-PEX5 bound to TALON resin. The resin was washed to remove unbound antibodies. Antibodies specific to $(His)_6$-PEX5 were eluted with 50% ethylene glycol [24].

2.7. SDS-PAGE and Western blotting

Polyacrylamide (8%) gel electrophoresis in the presence of 0.1% SDS (SDS-PAGE) was performed according to Laemmli [25]. After electrophoresis, the gels were either stained with Coomassie brilliant blue, or used for immunoblotting according to the method of Towbin [26]. The membranes were blocked by incubation in TBS [10 mM Tris/HCl (pH 8.0), 150 mM NaCl] containing 0.1% Tween 20 and 5% (w/v) lowfat milk powder. For detection of the respective proteins on Western blots, the primary antibodies were diluted (1:2000) in blocking solution. In addition to anti-PEX5, two other antisera, raised against HK purified from *T. brucei* [27] and against a peptide fragment of TbPEX11 [28] respectively, were also used. The secondary antibody, anti-rabbit horseradish peroxidase conjugated Ig (Rockland, USA), was diluted 1:25 000 and visualized with Lumi-light substrate (Boehringer Mannheim).

2.8. Protein determination and enzyme assay

Protein was measured according to Bradford, with bovine serum albumin as a standard [29]. The assay for glycosomal phosphoglycerate kinase-C (PGK) activity was performed as described [30].

2.9. $(His)_6$-PEX5 in vitro binding

Ten μg of $(His)_6$-PEX5 was coupled to 50 μl of TALON resin by incubation for 15 min at room temperature with mixing in 1 ml 100 mM triethanolamine/HCl (pH 8.0), 0.5 M NaCl, 10% glycerol. The affinity resin was washed thoroughly

Fig. 1. Amino-acid sequence alignment of *Trypanosoma brucei* (Tb), human (Hs) [38,49,50], tobacco (Nt) [51], yeast (Sc) [16] and *Pichia pastoris* (Pp) [17] peroxin 5 (PEX5). The alignment was performed using ClustalW [52] and graphically displayed using the ALSCRIPT program [53]. Hyphens indicate the absence of amino acids at corresponding positions. Numbering of amino-acid residues is according to the *T. brucei* sequence. The amino acids identical in all sequences are highlighted with a black background and positions with conserved substitutions in one or more sequences are shaded in gray. Tetratricopeptide repeat (TPR) repeats are indicated by horizontal lines above the alignment and numbered; the first residue of each repeat is indicated by an arrowhead. The W-X-X-X-F/Y pentapeptides are indicated in italics.

with the same buffer. Twenty μg of purified *T. brucei* PGK or truncated PGK [31] was incubated with the $(His)_6$-PEX5 resin in a final volume of 1 ml with gentle shaking for 60 min at room temperature followed by centrifugation of the suspension. The pellet containing the resin was washed with the same buffer as above, and subsequently incubated in the same buffer containing 5 mM EDTA to detach the proteins that were originally bound to the $(His)_6$-PEX5 resin. After centrifugation, the proteins present in the supernatant were precipitated with 10% (w/v) trichloroacetic acid and analyzed by SDS-PAGE.

For the determination of the binding constant, increasing amounts of purified *T. brucei* PGK were incubated under the same conditions as described above with the $(His)_6$-PEX5 resin. After centrifugation, the PGK activity was then measured in the two fractions.

2.10. Growth and isolation of the organism

Bloodstream forms of *T. brucei* stock 427 were grown in 300 g Wistar rats. Blood was collected from animals showing high parasitaemia by cardiac puncture under ether anaesthesia. Trypanosomes were harvested from the blood according to the method of Lanham [32]. Cells were washed three times in a buffer containing 0.25 M sucrose, 25 mM Tris/HCl (pH 7.8), 1 mM EDTA by centrifugation at $1000 \times g$ for 5 min at 4°C.

2.11. Subcellular fractionation

Washed trypanosome pellets were disrupted by grinding with silicon carbide in a buffer containing 0.25 M sucrose, 25 mM Tris/HCl (pH 7.8), 1 mM EDTA, 1 μM phenylmethylsulfonyl fluoride and 1 μM each leupeptin, pepstatin, E-64. Unbroken cells and nuclei were removed by centrifugation at $1500 \times g$ for 10 min at 4°C. The supernatant was further centrifuged at $5000 \times g$ for 10 min to pellet the granular fraction and then at $48\,000 \times g$ for 20 min. The pellet resulting from the last centrifugation is enriched in glycosomes and microsomes while the supernatant contains soluble proteins.

Digitonin fractionation of intact cells was performed as described [33].

2.12. Carbonate extraction of membranes

The one-step carbonate procedure as described by Fujiki et al. [34] was used to prepare a membraneous fraction containing only integral membrane proteins. Fractions containing purified glycosomes (15 mg protein ml^{-1}) were diluted 100-fold with ice-cold 100 mM sodium carbonate, pH 11.5, kept on ice for 30 min, and centrifuged for 60 min at $233\,000 \times g$ to separate the soluble fraction from the membrane pellet. Protein was precipitated from the supernatant by adding 10% (w/v) trichloroacetic acid.

2.13. Electron microscopy

Cells were fixed and prepared for electron microscopy and immunocytochemistry as described before [35]. Immunolabelling was performed on ultrathin sections of Unicryl-embedded cells, using anti-PEX5 antibodies and gold-conjugated goat-anti-rabbit antibodies.

3. Results

3.1. Cloning, sequencing and analysis of the T. brucei PEX5 gene

To facilitate the identification of the *PEX5* gene of *T. brucei*, a multiple sequence alignment was made of four yeast PEX5 protein sequences available in the databases [16–19]. Conserved regions were found and used to specify degenerate oligonucleotides for PCR amplification of gene segments. After cloning and sequencing, a 538 bp fragment was found coding for an amino-acid sequence that significantly resembled that of PEX5 of other organisms. The fragment was then used to screen a genomic library. A positive clone thus identified was sequenced revealing the presence of a long open-reading frame (ORF). However, its start codon could not unambiguously be determined because numerous in-frame ATG triplets were found at the 5′ side of the ORF.

The longest ORF codes for a polypeptide of 654 residues (excluding the putative initiator methionine) with a calculated molecular mass of 69 960 Da. Its predicted isoelectric point is 4.47 indicating a rather acidic protein. Using the genomic clones, as well as uncloned genomic DNA, a restriction map of the gene and its surrounding regions was constructed (data not shown). Only one locus containing a single gene could be detected in the *T. brucei* genome.

The *T. brucei* sequence was aligned with a representative subset from the 14 currently available PTS-1 receptor sequences of other organisms (Fig. 1). The overall identity of the putative *T. brucei* PEX5 with the other proteins is low (22–27%). The identity is considerably higher (31–40%) in the carboxyl-terminal half, which contains tetratricopeptide repeats (TPR). A TPR domain consists of several repeats of a degenerate 34-amino acid motif arranged in tandem array [36]. Like its homologue in other organisms, the *T. brucei* polypeptide contains seven repeats of the consensus motif in the C-terminal part. Repeats 1–3 and 5–7 fit well to the consensus, whereas repeat 4 is less well conserved as is the case in other organisms. In its N-terminal half, the *T. brucei* sequence contains 3 copies of the pentapeptide W-X-X-X-F/Y starting at positions 52, 200 and 318 of the sequence in Fig. 1. This pentapeptide is also found, albeit in different number and at different positions in human, tobacco and yeast PEX5. This motif has recently been shown to be responsible for the interaction of PEX5 with PEX14 [37]. From the alignment and the conservation of motifs it was inferred that the protein identified is indeed most likely the homologue of the PTS-1 receptor characterized in several other organisms.

It is noteworthy that a valine (codon GTG) is found at position 375 of the trypanosome PEX5 sequence. This position corresponds to the 3′ end of the sense primer used for amplification and which actually terminated in -GC, the first two nucleotides of an alanine codon. It is thus surprising that amplification could be achieved with this primer. Possibly, the primer was heterogeneous with respect to its 3′ end. Alternatively, *T. brucei* may be polymorphic with respect to its *PEX5* sequence, one allele coding for an alanine, whereas the other one that was cloned having a valine codon.

3.2. Overexpression and purification

A procedure was developed for the purification of recombinant *T. brucei* PEX5 from the soluble fraction of *E. coli* cells. The overexpressed protein comprises the 505 C-terminal amino acids of the 654 residues encoded by the ORF, and contains a $(His)_6$-tag fused to its N-terminus. Using immobilized metal affinity chromatography, the protein was purified to near-homogeneity as assessed by SDS-PAGE followed by Coomassie brilliant blue staining (data not shown).

The molecular mass of the bacterially expressed protein, as determined by SDS-PAGE, is approximately 67 000 Da. This value is larger than that calculated from the gene sequence (57 918 Da, including the spacer and tag). However, this difference could be attributed to anomalous migration during gel electrophoresis because a value of 57 960 Da was determined by mass spectrometry (A. Kumar and W. Hol, personal communication). Aberrant migration on SDS-PAGE has also been described for human, plant and *P. pastoris* PEX5, all proteins with a low isoelectric point [17,38,39].

3.3. PEX5 interacts in vitro with a PTS-1 containing protein

The ability of the purified $(His)_6$-PEX5 to interact in vitro with a PTS-1 containing protein, the glycosomal isoenzyme of phosphoglycerate kinase (PGK-C) of *T. brucei* was tested. Purified PGK-C was mixed with recombinant $(His)_6$-PEX5 and subjected to metal affinity chromatography. As shown in Fig. 2, the two proteins were eluted together upon addition of EDTA. When the experiment was performed with a truncated form of PGK-C [31], lacking the C-terminal 20 amino acids and hence the PTS-1 signal, the latter did not bind to the $(His)_6$-PEX5 column and thus was not eluted together with TbPEX5. This indicates that the interaction between PEX5 and PGK-C depends specifically on the PTS-1 containing C-

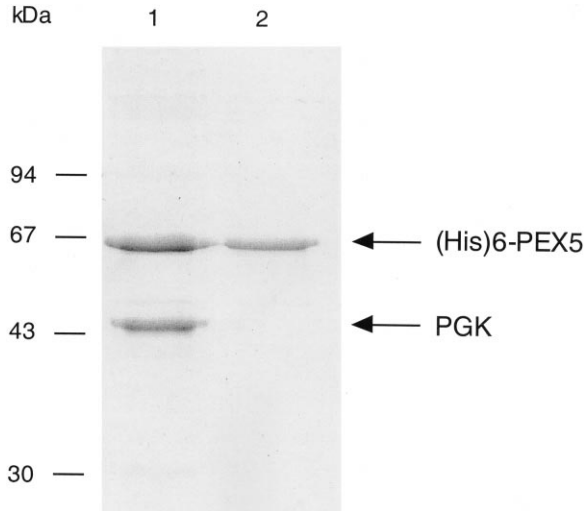

Fig. 2. Binding of glycosomal phosphoglycerate kinase-C (PGK-C) to *Trypanosoma brucei* PTS-1 receptor protein (TbPEX5). TbPEX5 was modified at its N-terminus by the addition of a $(His)_6$-affinity tag. The protein, overexpressed in *Escherichia coli*, was purified using immobilized metal affinity chromatography. Purified $(His)_6$-peroxin 5 (PEX5) was incubated with full-length glycosomal PGK (lane 1) or with a truncated PGK lacking its PTS-1 (lane 2) as described in Section 2.

terminal extension and thus provides further confirmation of the identity of the cloned protein as a PTS-1 receptor.

The binding of PGK-C to $(His)_6$-PEX5 is saturable. Using Scatchard analysis it was established that PGK-C has a high affinity for its receptor; the equilibrium dissociation constant (K_d) has a value of 40 nM (Fig. 3).

3.4. Expression of the PEX5 gene in T. brucei

A probe comprising the 5′ half coding region of the *PEX5* gene was hybridized with a Northern blot containing total RNA isolated from bloodstream and procyclic forms of *T. brucei*. A transcript of approximately 3600 nucleotides was detected at similar levels in both stages of the life cycle (Fig. 4A). A diffuse band of higher molecular mass transcripts was also observed, the significance of which is unknown.

Antibodies raised against the recombinant protein detected a protein with an apparent

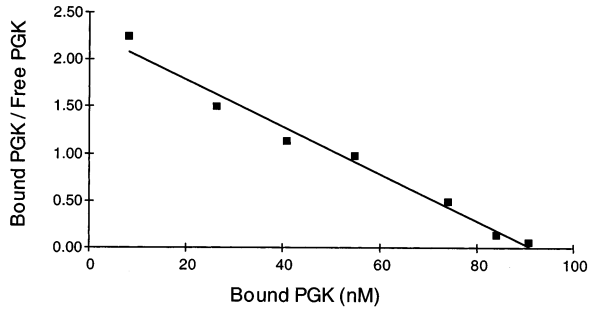

Fig. 3. Quantitative analysis of the binding of glycosomal phosphoglycerate kinase-C (PGK-C) to *Trypanosoma brucei* PTS-1 receptor protein (TbPEX5). Increasing concentrations of full-length glycosomal PGK were incubated with $(His)_6$-peroxin 5 (PEX5) in an in vitro binding assay as described in Section 2. The results are presented as a Scatchard plot to allow the determination of the equilibrium dissociation constant (K_d).

molecular mass of 90 kDa in total cell extracts prepared from bloodstream and procyclic forms of *T. brucei* (Fig. 4B). Again, the molecular mass

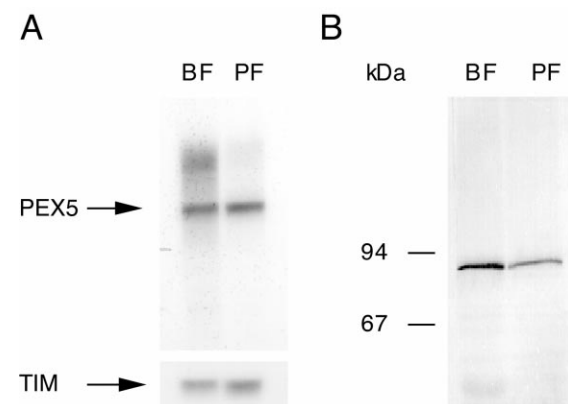

Fig. 4. Peroxin 5 (PEX5) expression in *Trypanosoma brucei*. (A) Northern blot analysis of total RNA isolated from bloodstream form (BF) or procyclic form (PF) *T. brucei*. Equal amounts of both RNA preparations were loaded on the gel. The filter was hybridized with gene-specific probes as indicated in the figure. Triosephosphate isomerase (TIM) was used as control because the gene is equally expressed in both stages of the trypanosome life cycle [54]. (B) Equal amounts of protein extracts from bloodstream and procyclic forms were separated by SDS-PAGE and transferred to nitrocellulose membranes. PEX5, with an apparent molecular mass of 90 kDa, was detected using antibodies raised against the recombinant $(His)_6$-*T. brucei* PTS-1 receptor protein (TbPEX5).

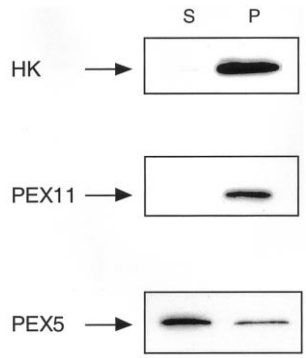

Fig. 5. Subcellular localization of peroxin 5 (PEX5). A bloodstream form *Trypanosoma brucei* homogenate was separated by differential centrifugation into a cytosolic (S) and organellar (P) fraction as described. The fractions were analyzed by SDS-PAGE and Western blotting using antibodies against hexokinase (HK), PEX11 and PEX5. Only the relevant portions of the blot are shown.

of this protein appeared to be higher than that calculated from the protein sequence due to the abnormal migration of PEX5 proteins in SDS-PAGE. The PTS-1 receptor is thus expressed to similar levels in both stages of the life cycle of the parasite.

3.5. Subcellular distribution

A *T. brucei* cell homogenate was fractionated by differential centrifugation into a soluble fraction, corresponding to the cytosol, and an organellar fraction enriched in glycosomes. The subcellular distribution of PEX5 and marker enzymes was examined by immunoblot analysis of these different fractions (Fig. 5). Virtually no HK, a marker enzyme of the glycosomal matrix [27], was found in the cytosolic fraction indicating that glycosomes were apparently not damaged during the isolation procedure. PEX5 was detected both in the cytosol and in the glycosome-enriched fraction. About 25% of PEX5 was located in the glycosome as estimated from a Western blot, on which an equal amount of protein from the glycosomal and cytosolic fractions had been loaded. Since it is known that the total amount of proteins in the glycosomal compartment is 6 times less than in the cytosol [40], the steady-state level of PEX5 associated with glycosomes must be low, probably less than 5% of the total quantity in the cell. The association of only a small quantity of PEX5 with the glycosomes was confirmed by a similar analysis performed with glycosomes purified by sucrose gradient centrifugation (not shown).

Additional evidence that PEX5 has a predominantly cytosolic localization in trypanosomes was derived from fractionation of cells after membrane permeabilization with digitonin. Most PEX5 was already released at very low digitonin concentrations [25–75 μg digitonin (mg protein)$^{-1}$], a behaviour consistent with a cytosolic location of the protein (Fig. 6). The glycosomal marker HK was only released at much higher concentrations of the detergent [higher than 225 μg digitonin (mg protein)$^{-1}$].

The nature and extent of the association of PEX5 with glycosomes was further analyzed by treating purified glycosomes with carbonate (Fig. 7). The matrix enzyme HK was mainly present in the soluble fraction of the organelle while PEX11, an integral membrane protein [28] was present in the membraneous fraction. Most of the PEX5 found associated with the purified glycosomes was released in the soluble fraction upon carbonate treatment, indicating a weak or no association with the glycosomal membrane.

In immunocytochemical experiments, using anti-TbPEX5 antibodies on ultrathin sections of *T. brucei* a cytosolic labelling was observed; occasionally also some labelling of glycosomes was seen (not shown). This is consistent with the biochemical data indicating that only a small

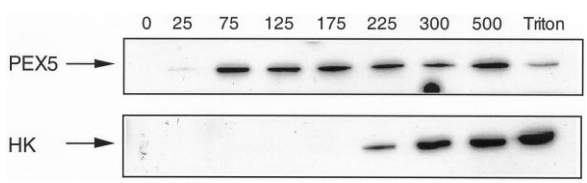

Fig. 6. Subcellular localisation of peroxin 5 (PEX5) by digitonin titration. The digitonin soluble fraction was subjected to Western blot analysis. The quantity of digitonin [μg (mg protein)$^{-1}$] used to treat the cells is indicated above the lanes. Triton indicates that the cells have been treated with 0.2% Triton X-100. Hexokinase is used as marker enzyme for the glycosomal compartment.

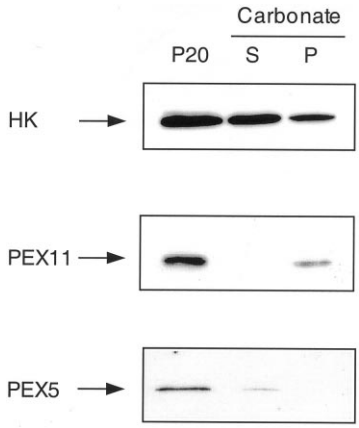

Fig. 7. Subperoxisomal localisation of peroxin 5 (PEX5). A 48 000 × *g* pellet (P20) containing organellar material was prepared from bloodstream form *Trypanosoma brucei* and treated with carbonate at pH 11.0. After centrifugation a membrane pellet and a soluble fraction were obtained. Equal proportions of pellet (P) and supernatant (S) fractions were subjected to SDS-PAGE and blotted to nitrocellulose filters. Antisera specific of hexokinase (HK), PEX11 and PEX5 were used to visualize the distribution of the proteins.

quantity of PEX5 is associated with the organelles.

4. Discussion

In this paper evidence is provided for the identification of TbPEX5, the trypanosomal homologue of the PTS-1 receptor as characterized in several yeasts and human. The overall sequence similarity between TbPEX5 and its various counterparts is rather low. Yet, the trypanosome protein contains the seven TPR repeats present in all PTS-1 receptors identified so far. Over ten classes of proteins present in organisms ranging from human to bacteria have been shown to contain TPR motifs. These proteins are involved in a variety of diverse functions including cell-cycle regulation, neurogenesis, protein-kinase inhibition, protein folding, protein transport across mitochondrial and peroxisomal membranes among others. TPR motifs have been shown to mediate protein–protein interactions [36]. In PEX5s the TPR motifs are responsible for the interaction with PTS-1 containing proteins or peptides. This was shown both in vitro [41] and, using the two-hybrid system, in vivo [42]. TbPEX5 also contains three copies of the pentapeptide W-X-X-X-F/Y characteristic of PEX5s.

The conservation in Kinetoplastida of genes (*PEX2*, *PEX11* and, as presented here, *PEX5* as well) involved in peroxisomes biogenesis also lends further strong support for the notion of a common evolutionary origin of peroxisome and glycosome [1,43].

It has been shown that purified recombinant TbPEX5 is able to bind specifically and with high affinity *T. brucei* PGK-C, a PTS-1 containing protein. The equilibrium dissociation constant of 40 nM that was determined is 12.5 times lower than that reported for the interaction between *P. pastoris* PEX5 and a PTS-1 containing peptide [41]. One could wonder if this higher affinity is due to the fact that we measured the interaction of PEX5 with a full-length protein rather than with a peptide. Indeed, it has been shown that the sequence adjacent to the C-terminal tripeptide modulates the efficiency of peroxisomal transport at least for specific proteins [11,44,45]. The high affinity of PEX5 for its ligand is probably a prerequisite for efficient recognition and binding of newly synthesized proteins with a PTS-1 in the cytosol. This result is also in line with the notion that PTS-1 receptors of different organisms may recognize a different range of sequences. The glycosomal *T. brucei* PGK, ending with the C-terminal tripeptide-SSL, is not imported into peroxisomes when it is expressed in mammalian cells [11]. Recently it was demonstrated in a two-hybrid screen that a peptide ending in -SSL does not interact with *S. cerevisiae* PEX5 and only weakly with human PEX5 [44].

On Northern and Western blots comparable levels of transcript and protein, respectively, were observed in the bloodstream and procyclic form of the parasite. This argues for the importance of the glycosome in both stages of the life cycle. Indeed, all attempts to generate a double knock-out of PEX2 or PEX11, the two other proteins involved in glycosome assembly identified to date, by disrupting their genes have failed so far [28,46]. From Southern blotting, we infer that *TbPEX5* is a single-copy gene. The presence of multiple in-

frame ATG triplets in the 5′ region of the gene precluded so far the identification of the start codon.

Data obtained by immunocytochemical analysis and various biochemical approaches point to a predominantly cytosolic localization of PEX5 with a minor portion of the protein associated with glycosomes. This is consistent with the hypothesis of receptor cycling between the cytosol and peroxisomes [15]. It remains to be established if the PEX5 associated with glycosomes is located in the matrix. This would enable one to distinguish between the two alternative hypotheses implying a 'short shuttle' or an 'extended shuttle' mechanism, respectively. According to the first hypothesis, PEX5 binds to the membrane-bound receptors PEX13 and PEX14. PEX5 releases then its PTS-1 cargo at the membrane surface for subsequent translocation into the peroxisome matrix. In the second hypothesis, PEX5 binds to PEX13 and PEX14 but does not release the PTS-1 cargo until both PEX5 and the PTS-1 protein arrive in the matrix. It has been postulated that the ubiquitin-conjugating protein PEX4 may be involved in the recycling of PEX5 molecules to the cytosol [47].

Finally, the identification of the trypanosomal PTS-1 receptor will allow one to screen for compounds that may inhibit the interaction between the receptor and its ligand. This inhibition should be selective for the parasite glycosomes, such that PTS-1 containing proteins of the human host can still interact with their PEX5 and be imported. The predicted strong decrease in the rate of glycosomal protein import resulting from such an inhibition would lead to the mislocalization of the glycolytic enzymes possessing a PTS-1. Such a mislocalization has already been shown to be very detrimental to the survival of trypanosomes [48].

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